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A Study of the Peripheral Blood of a Metamorphosing Anuran (*Rana pipiens*).

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The blood of the adult anuran has been extensively investigated by Jordan and his co-workers. These studies have resulted in a rather complete understanding of adult anuran hematology.

The larval anuran, however, although a fairly common laboratory form, has been studied far less intensely. Maximow (1910) and Jordan and Speidel (1923) have described changes in hematopoietic centers during development from the larval to the adult form. McCutcheon (1936) suggests a possible phylogenetic change in the hemoglobin molecule of the metamorphosing *Rana catesbeiana.* Jordan and Speidel (1922-23) designate definite functions to the leucocytes during the metamorphic process. These workers claim the lymphocyte is concerned with the progressive changes and the granulocytes with the regressive changes of metamorphosis. According to these findings it is possible that a systematic study of the circulating blood throughout metamorphosis may clarify the role of the blood in the process.

It is the purpose of this investigation to study the circulating blood of the metamorphosing anuran (*Rana pipiens*) in order to determine whatever changes occur, and how they may be related to definite stages of metamorphosis.

Material and Methods.

The *Rana pipiens* larvae were procured by Rugh's (1934) method of ovulation and fertilization. The larvae were placed in finger bowls in 1/10 Holtfreter's solution until hatching and then were transferred to aerated six-inch high tanks. The animals were fed solely on boiled spinach.

The following stages[2] were utilized:

I. 11-13 mm. total length
II. 15-16 mm. total length } Pre-metamorphic stages.
III. 20-22 mm. total length
IV. Hind-limb bud length.
V. 3 mm. hind-limb bud.
VI. Fully differentiated hind limb.
VII. 18 mm. hind limb.
VIII. Fore-limb emergence.
IX. Tail resorption.
X. Newly-metamorphosed adult.

The animals were anesthetized by being placed in a chloretone solution. The heart was exposed and blood was drawn directly from the ventricle by means of a micropipette previously rinsed in a dilute sodium citrate solution. All operations were accomplished with the aid of a dissecting microscope.

Smears were obtained by spreading the drop with a cover slip. In the earlier stages it was impossible to make smears due not only to the small amount of blood obtained but also because of its dilute nature, i.e., scarcity of cellular elements. Wright's stain was used exclusively for all smears.

Four cell types were counted in making the differential count—lymphocytes, eosinophiles, neutrophiles and thrombocytes. Four hundred cells were counted on each slide; two hundred on one end of the smear and two hundred on the other end. Differential cell counts were not feasible in stages earlier than the 3 mm. hind-limb bud stage.

Observations and Results.

Stage I (11-13 mm.).

The erythrocyte, lymphocyte, thrombocyte and haemocytoblast are the only discernible elements present. No granulocytes were observed at this stage.

The Erythrocyte: This early stage contains many primitive erythrocytes. They range in shape from a small spherical cell with a large ovoid nucleus, to the definitive erythrocyte possessing an ovoid shape and proportionately, a smaller ovoid nucleus. There are many intermediate cells between these two extremes.

Many of the more primitive cells contain a basophilic cytoplasm and transition stages are present which may denote derivations from haemocytoblasts which are present in the circulating blood.

The cytoplasm of the red blood cells is

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[2] The stages of metamorphosis are the same as those used by D'Angelo and Charipper, 1939.

vacuolated and contains large numbers of yolk granules. The vacuoles are of varying sizes and are unevenly distributed. There may be only one or two vacuoles or at times a sufficient number as to make the cytoplasm appear alveolar.

Haemocytoblasts are present in rather large numbers in this early stage. These cells conform in all respects to the classical description. In addition they are often vacuolated, and always contain yolk granules.

Typical lymphocytes and thrombocytes are present at this stage.

Three processes are occurring in the erythrocyte at this stage, and to a varying extent persist throughout the rest of the stages of metamorphosis. They are erythroplastid formation, amitosis and mitosis. The erythroplastid formation appears to be similar in all details to what was described by Dawson (1928). Amitosis follows precisely the course described by Charipper and Dawson (1928). Mitosis is typical and all phases of this method of reproduction can be observed.

Stage II (15-16 mm.).

In the 15-16 mm. tadpole the granulocytes are first observed in very small numbers.

The neutrophile is represented by both adult types (typical granulation and polymorphous nucleation) and embryonic types (typical granulation and single or bilobed nucleus).

The eosinophiles are largely embryonic, consisting of a single nucleus and a small number of typical eosinophile granules.

In all other respects the condition of the other elements is identical with the previous stage.

Stage III (20-22 mm.).

The erythrocyte contains less yolk granules and the normoblasts have decreased in number. Haemocytoblasts are still much in evidence. Eosinophiles are still largely immature, the neutrophiles are the same as in the previous stage; mitosis and amitosis occur but with less frequency; erythroplastids persist.

A few atypical erythrocytes are first seen at this stage. They are large cells, which may be vacuolated, containing a large lilac-staining nucleus and a faintly staining cytoplasm, whose periphery is not always discernible. These cells resemble the senile erythrocyte described in the adult by Jordan (1919).

Stages IV through X.

Metamorphosis.

The following stages of metamorphosis introduce no radical changes, except in differential counts. Therefore their gross changes will be discussed together.

The Erythocyte: The vacuolization decreases with the approach of metamorphosis as does the presence of yolk granules in the cytoplasm.

Five types of erythrocytes are observed throughout the active transformation.

1. The normoblast, although in far smaller numbers.
2. The small erythrocyte with the polarized nucleus.
3. The large erythrocyte.
4. The normal ovoid cell.
5. The senile erythrocyte.

Haemocytoblasts are present in smaller number and in consequence transition forms are less frequently encountered than in the early stages.

Large, medium and small lymphocytes are present, as are the typical thrombocytes. The eosinophiles are observed in small numbers and in the later stages they have assumed the typical adult histology. Transitional stages from the uninuclear neutrophile to the adult polymorphonuclear neutrophile are observed throughout metamorphosis.

Although mitosis and amitosis continue throughout the larval stages and the entire process of metamorphosis and beyond to the newly emerged adult, there is a decrease in both types of intra-vascular reproduction with the onset of the metamorphosis. In the differentiated hind-limb stage there appears to be an increase in mitosis and in the newly-emerged adult amitosis is more prevalent than mitosis, which occurs rarely.

Stages V through X.

The Differential Counts.

The results of the differential counts are summarized in Table I and graphically recorded in Table II.

The lymphocyte is present in greatest numbers in the early part of metamorphosis (Stages V, VI, VII), the greatest number being present at the differentiated hind-limb stage (VI). The amount decreases until the lowest point is reached at the period of tail resorption (Stage IX) and it rises again in the newly-emerged adult (Stage X).

The percentages of thrombocytes follow a precisely opposite course to that of the lymphocytes, except in the fore-limb stage (VIII) where a significant increase in the neutrophiles is observed.

The first significant change in the neutrophile count is observed in the increase in the fore-limb stage (VIII). The amount is slightly decreased in the period of tail resorption, and attains the lowest percentage in the newly-emerged adult. The eosinophiles are never present in large numbers. The counts range from .86% in the newly-emerged animal to 3.1% in the 18 mm. hind-limb length stage and at no time was a significant change recorded.

DISCUSSION.

The most outstanding process occurring in the larval stages, and continuing through-

TABLE I.

Stage	Lymphocyte %	Standard Error	Thrombocyte %	Standard Error	Neutrophile %	Standard Error	Eosinophile %
3 mm. Hind-limb Bud	56.5	±4.2	38.5	±2.26	4.0	±2.10	1.27
Diff. Hind-limb Bud	70.4	±2.84	21.74	±2.99	5.41	±1.92	1.0
18 mm. Hind-limb Length	60.45	±2.73	30.06	±2.6	6.9	±1.11	3.1
Fore-limb Emergence	53.43	±3.2	31.65	±3.1	12.21	±2.98	1.7
Tail Resorption	46.41	±3.09	45.9	± .72	7.71	±1.29	1.25
Newly-Metamorphosed Adult	58.74		37.3		3.4		.86

out the entire transformation to a decreasing extent, is that of erythropoiesis in the circulating blood. Although erythropoiesis in the general circulation has never been reported in the adult or larval *Rana pipiens*, it has been observed in many other forms.

Jordan and Speidel (1930b) describe erythropoiesis in the circulating blood of the adult cyclostome. Maximow (1910) in *Rana temporaria* and Ichikawa (1934) working with urodeles find the first erythropoietic loci is distributed throughout the peripheral circulation. Upon splenectomy of the adult *T. viridescens*, Jordan and Speidel (1930a) observed a shift of erythropoiesis to the peripheral circulation. Erythropoiesis also has been described in the circulating blood of the embryonic turtle (Jordan and Flippen, 1913).

This evidence may indicate that the peripheral circulation is a primitive center of erythropoiesis. It is observed in the adult cyclostome, and is only retained in the embryonic and larval stages of some amphibians and reptiles.

Though this investigation does not include an examination of any other possible centers of erythropoiesis, the marked decrease in red cell production in the peripheral blood with the progress of metamorphosis suggests this important function may be more fully assumed elsewhere.

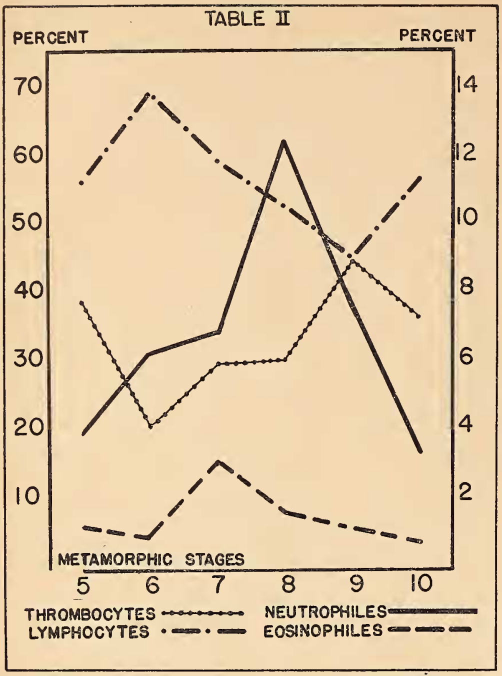
Mitosis and amitosis have never been reported in the circulating blood of the mature adult or larval *Rana pipiens*. However, Dawson (1930) has described mitosis in urodeles, and Charipper and Dawson (1928) have reported amitosis in the peripheral circulation of *Necturus*. The peripheral circulation of the embryonic turtle (Jordan and Flippen, 1913) and the larval *Lepidosiren* (Bryce, 1906) possesses both these methods of reproduction.

The presence of vacuoles (more predominant in the larval stages) in the erythrocyte were also observed by Bryce (1906) in the larval *Lepidosiren* and by Dawson (1931) in the blood of adult *Necturus*. The senile cell observed here was also reported by Jordan and Flippen (1913) and Jordan (1919). It occurs in preponderant numbers in the later stages of metamorphosis.

It is significant to note that the lymphocytes are more numerous in the early stages of metamorphosis, reaching their maximum at the differentiated hind-limb stage (VI), and decreasing for the duration of the transition, to rise again in the newly-emerged adult, whereas the neutrophiles attain their highest significant percentage in the fore-limb stage (VIII).

From statistics on growth throughout active metamorphosis supplied by D'Angelo, Gordon and Charipper (1941), the height of active growth occurs at the early stages of active metamorphosis, and the beginning of regressive changes (changes from the larval body to the adult form, loss of tail, etc.) occurs at fore-limb emergence and reaches its height at tail resorption.

The above two relationships may indicate that the lymphocyte is concerned with progressive changes and the neutrophile with regressive changes. This hypothesis agrees with Jordan and Speidel's (1922-23) conclusion, which maintained the lymphocyte is



responsible for the progressive changes, or growth, during metamorphosis, and regressive changes are caused by granulocytes. The neutrophiles are believed to play an important role in the resorption of the tail, and the eosinophiles are vital for the transformation of the intestine. Grant (1931) on examination, histologically, of *Urodele* larvae, reported an eosinophilia to be present. However, no significant change was observed in this investigation.

On the whole, the thrombocyte's percentages seem to be in direct antithesis to that of the lymphocyte. This fluctuation may be explained by its mode of origin. It is generally accepted that the thrombocyte arises from the lymphocyte, particularly the small lymphocyte. Therefore, it is possible that in the active stages of growth the demand for the lymphocytes is so urgent that they are utilized for progressive changes rather than as progenitors of the thrombocyte.

Conclusions.

I. Erythropoiesis has been found to occur in the circulating blood of the metamorphosing *Rana pipiens*. This phenomenon is more pronounced in the early stages.

II. The erythrocytes in the early larval stages are characterized by cytoplasmic vacuoles and yolk granules, which are progressively lost as metamorphosis proceeds. The presence of four types of erythrocytes in the adult *Rana pipiens* reported by Jordan (1919) is confirmed in the later stages of metamorphosis.

III. Maturation of granulocytes has been observed in the circulating blood throughout the metamorphic process.

IV. The concentration of the thrombocytes, in general, varies inversely with those of the lymphocytes. The eosinophiles show a constant low percentage throughout the entire metamorphic process.

V. During metamorphosis the concentration of lymphocytes and neutrophiles fluctuates. In the early stages of this process, the period of most active growth, the concentration of lymphocytes is relatively high, and the neutrophile percentage remains constant, whereas, in the later stages of transformation, which are characterized by regressive changes, the lymphocytes undergo a marked decrease in amount and the neutrophiles demonstrate a significant increase.

VI. These results support the contention of previous investigators (Jordan and Speidel, 1922, 1923) who believe the lymphocyte is associated with progressive growth and the neutrophiles with regressive changes of the metamorphosing anuran.

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